In Vivo Validation of PAPSS1 (3′-phosphoadenosine 5′-phosphosulfate synthase 1) as a Cisplatin-sensitizing Therapeutic Target

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Abstract

Purpose: Our previous screening efforts found that inhibition of PAPSS1 increases the potency of DNA-damaging agents in non–small cell lung cancer (NSCLC) cell lines. Here, we explored the clinical relevance of PAPSS1 and further investigated it as a therapeutic target in preclinical model systems.

Experimental Design: PAPSS1 expression and cisplatin IC50 values were assessed in 52 lung adenocarcinoma cell lines. Effects of PAPSS1 inhibition on A549 cisplatin sensitivity under hypoxic and starvation conditions, in 3D spheroids, as well as in zebrafish and mouse xenografts, were evaluated. Finally, the association between PAPSS1 expression levels and survival in patients treated with standard chemotherapy was assessed.

Results: Our results show a positive correlation between low PAPSS1 expression and increased cisplatin sensitivity in lung adenocarcinoma. In vitro, the potentiation effect was greatest when A549 cells were serum-starved under hypoxic conditions. When treated with low-dose cisplatin, PAPSS1-deficient A549 spheroids showed a 58% reduction in size compared with control cells. In vivo, PAPSS1 suppression and low-dose cisplatin treatment inhibited proliferation of lung tumor cells in zebrafish xenografts and significantly delayed development of subcutaneous tumors in mice. Clinical data suggest that NSCLC and ovarian cancer patients with low PAPSS1 expression survive longer following platinum-based chemotherapy.

Conclusions: These results suggest that PAPSS1 inhibition enhances cisplatin activity in multiple preclinical model systems and that low PAPSS1 expression may serve as a biomarker for platinum sensitivity in cancer patients. Developing strategies to target PAPSS1 activity in conjunction with platinum-based chemotherapy may offer an approach to improving treatment outcomes.

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Introduction

The majority of non–small cell lung cancer (NSCLC) patients are diagnosed with an inoperable disease and are given platinum-based chemotherapy or molecularly targeted treatments.

Platinum-based regimens have been the mainstay treatment for NSCLC for over 30 years with response rates of 30%–33% (1). Although some patients may benefit from targeted therapies, treatment resistance almost inevitably emerges and for these patients, platinum-based chemotherapy is the next line of treatment (2). Because of the ongoing clinical problem of tumor heterogeneity (3–5) and the fact that platinum-based regimens have provided patients with meaningful therapeutic benefits, platinum-based anticancer drugs (platins) are unlikely to be replaced as standard of care in the near future. One way to improve treatment outcomes for patients with advanced NSCLC is to define strategies that will enhance the activity of platins. Depending on the tumor microenvironment, some cell populations within the tumor will be exposed to sublethal doses of chemotherapy, contributing to poor response and eventually, tumor recurrence. If these cells were to be sensitized to platins, then they would not be able to survive even suboptimal doses of the treatment and contribute to the development of drug-resistant tumors.

We have previously validated that PAPSS1 knockdown sensitizes four different NSCLC cell lines (A549, H460, H358, and H1703) to cisplatin treatment with no specificity for histologic subtype or driver mutation (6). We have also demonstrated that PAPSS1 inhibition sensitizes NSCLC cells not only to platins, but also to other DNA-damaging cancer treatments including topoisomerase I inhibitors and radiation. Here, we first analyzed...
PAPSS1 expression and their corresponding cisplatin IC50 in 52 lung adenocarcinoma cell lines to validate and extend our previous findings suggesting that PAPSS1 expression levels may be associated with cisplatin treatment outcome (6). With regards to developing PAPSS1 as a therapeutic target, we wanted to establish that PAPSS1 depletion sensitizes cells to cisplatin under different microenvironmental contexts typical of an in situ cancer setting. This is particularly important because tumor cells that are exposed to low doses of chemotherapeutics in vivo often have restricted access to oxygen and nutrients due to their large distances from blood vessels, impaired vascular systems, or changing metabolic requirements (7). We determined the therapeutic value of targeting PAPSS1 as a cisplatin modulator in vitro by testing the effects of PAPSS1 knockdown on cisplatin activity in A549 cells subjected to serum starvation and chronic hypoxia. We then provide further support that PAPSS1 is a target that should be pursued to improve the efficacy of platinum-based agents through three different in vitro and in vivo model systems: 3D spheroids and xenografts in zebra fish and mice. Existing clinical data on NSCLC and ovarian cancer patients further support that PAPSS1 is a clinically relevant target as low expression of this gene is associated with improved overall and progression-free survival, respectively, following platinum-based chemotherapy.

Materials and Methods

Associating cisplatin sensitivity with PAPSS1 expression in lung adenocarcinoma cell lines

Cisplatin IC50 values and corresponding RNA normalized gene expression microarray profiles for 52 lung adenocarcinoma cell lines were downloaded from the Wellcome Trust Sanger Institutes Genomics of Drug Sensitivity in Cancer website (http://www.cancerxgene.org/; refs. 8, 9). PAPSS1 expression (log2 intensity) and cisplatin IC50 values (log2) were aligned for each cell line and then separated into two groups, high and low PAPSS1 expression, using the average PAPSS1 expression across all cell lines as a cutoff. Resulting cisplatin IC50 values were compared between the two groups using the Mann–Whitney U test. Pearson correlation of cisplatin IC50 and PAPSS1 expression was also determined across all cell lines. One-tailed P values were determined with P < 0.05 considered significant. Similar analyses were performed using cisplatin AUC values and PAPSS1 expression data for 58 lung adenocarcinoma cell lines downloaded from the Cancer Target Discovery and Development (CTD2) Network (https:// ogc.cancer.gov/programs/ctd2/data-portal) established by the National Cancer Institute's Office of Cancer Genomics and profiled as part of The Cancer Therapeutics Response Portal v2 (https://portals.broadinstitute.org/ctrp.v2.1/; refs. 10–12).

Cell culture and reagents

A549 cells were obtained directly from Dr. John Minna (Dallas, TX) and cultured in RPMI1640 (11875119 Thermo Fisher Scientific) supplemented with 2 mmol/L L-glutamine (25300081 Thermo Fisher Scientific) and 10% FBS (12483020 Thermo Fisher Scientific). A549 NucLight Red cells were purchased from Essen Bioscience (4491) and maintained in Ham F-12 Nutrient Mix with GlutaMAX medium (31765092 Thermo Fisher Scientific) supplemented with 10% FBS and 2 mmol/L L-glutamine. Red fluorescence was sustained with 0.5 μg/mL puromycin (631306 Clontech Laboratories) until the start of an experiment. In vitro studies were performed using cells cultured between passages 3 and 15. All cell lines were tested mycoplasma-negative through the manufacturer or IDEXX BioResearch (frozen stocks were last tested January 2014) prior to experiments. PAPSS1 (1:1,000) primary antibody was obtained from Abcam (ab56398) while β-actin (1:50,000) primary antibody was purchased from Cell Signaling Technology (3700). Ready-to-inject cisplatin was obtained from Hospira.

siRNA transfections

Cells were seeded in 6-well plates and transfected with a pool of three siRNA duplexes targeting PAPSS1 (HSS113394, HSS189820, HSS189821; Thermo Fisher Scientific) as described previously (6). Please refer to the Supplementary Data for detailed methods.

Starvation cell viability assays

Cells were reverse transfected with siRNAs in 384-well plates (781091 Greiner Bio-One). At 30 hours posttransfection, the media were replaced with serum-free culture media. The following day, the cells were treated with different concentrations of cisplatin. At 48 and 72 hours postdrug treatment, cytotoxicity was assessed using a fluorescence-based metabolic assay, PrestoBlue (A13262 Thermo Fisher Scientific), as per the manufacturer’s instructions. Cells were incubated with PrestoBlue at 37°C for 1 hour. Metabolic activity was measured using FLUOstar OPTIMA microplate reader (BMG Labtech).

Hypoxia studies

Cells were reverse transfected (cells seeded and transfected simultaneously) and transferred to an incubator inside a hypoxic chamber (HYGB42 Coy Lab Products) with 1.0% oxygen at 30 hours posttransfection. The following day, drug dilutions were prepared with hypoxic media. At 48 and 72 hours following drug treatment, cell viability was assessed using the PrestoBlue reagent.

Translational Relevance

Lung cancer is the leading cause of cancer-related mortality with most patients diagnosed with inoperable disease. Despite the emergence of targeted therapy, 5-year survival rates remain <20% for non–small cell lung cancer (NSCLC) and there remains a strong reliance on platinum-based chemotherapy. Therefore, identifying ways to increase the effectiveness of platinum regimes has great potential to improve patient outcomes. We previously identified PAPSS1 as a protein that when inhibited, augments cisplatin activity in NSCLC cells. Here, we demonstrate that targeting PAPSS1 in conjunction with cisplatin treatment leads to better response rates in vivo. Clinically, we discovered that low PAPSS1 expression is associated with increased survival rates in chemotherapy-treated NSCLC and ovarian cancer patients. Our results provide new evidence that PAPSS1 expression/copy number could be predictive of treatment response to cisplatin. Furthermore, patients with high PAPSS1 expression/copy number may benefit from PAPSS1 inhibition in combination with platinum-based chemotherapy.
Spheroid assays

NucLight red A549 cells were transfected with PAPSS1-targeting siRNAs in 6-well plates as described previously (6). Cells were trypsinized 48 hours later and seeded in 96-well round bottom ultra-low attachment plates (89089-826 Corning) at 2,500 cells/well for spheroid formation. The plates were centrifuged (room temperature, 1,000 rpm, 10 minutes) and then imaged every 3 hours using the IncuCyte ZOOM Live Cell Imaging system (Esen BioScience). Three days later, the spheroids were treated with cisplatin and 62.5 nM SYTOX Green nucleic acid stain (S7020 Thermo Fisher Scientific) was added as a dead cell marker. Spheroids were imaged every 3 hours for 8 days and analyzed using the IncuCyte ZOOM software.

SDS-PAGE and Western blot analysis

All buffer chemicals were obtained from Sigma-Aldrich. Approximately 1 × 10^7 cells were lysed with lysis buffer (6). Cellular lysates were subsequently clarified (14,000 rpm, 20 minutes). Tumor samples (100–200 mg) were homogenized using FastPrep-24 instrument (116004500 MP Biomedicals) at 6 m/s for 30 seconds for at least 3 cycles with 5 minutes of rest between cycles until the tumor was dissolved. Further details on tissue homogenization are provided in the Supplementary Data. The homogenized lysates were collected by centrifugation (4°C, 14,000 rpm, 10 minutes). All samples were processed and probed for PAPSS1 expression as described previously (6). Protein levels were quantified using the ImageLab software.

Zebrafish husbandry

The transparent Casper strain (13) of zebrafish was maintained according to standard protocols (14). All zebrafish studies were approved by the Dalhousie University Committee on Laboratory Animals, Protocol #15-126 and performed in accordance with the Canadian Council of Animal Care (CCAC).

A549 cell staining and injection and screening of zebrafish

A549 cells with or without siRNA knockdown of PAPSS1 were stained with CM-Dil (C7001 Thermo Fisher Scientific) and inoculated as described previously (ref. 15; see Supplementary Data for details). At 48 hours postfertilization (hpf), approximately 150–200 A549 cells were injected into the yolk sac of each naturally dechorionated Casper embryo. The embryos were kept at 28°C for 30 minutes and subsequently at 35°C. Embryos with a fluorescent cell mass within the yolk sac at 12–24 hours postinjection (hpi) were selected and randomized for the experiments.

Live microscopy of zebrafish embryos

Every 24 hours for approximately 7 days, 4–6 embryos per experimental group were imaged and analyzed for cellular interactions within the zebrafish embryonic microenvironment. An inverted Axio Observer Z1 microscope equipped with a Colibri LED light source and an AxioCam Rev 3.0 CCD camera and Axiovision Rel 4.0 software (Carl Zeiss Microimaging Inc.) was used to screen, observe, and capture images of injected embryos.

Quantifying A549 cellular proliferation using the zebrafish

Injected embryos were placed into groups of 15–20 embryos and euthanized by a Tricaine (1 mg/mL; E10521 Sigma) overdose at 48 and 96 hpi and dissociated as described previously (15). The dissociations were analyzed using the inverted Axio Observer Z1 microscope (Carl Zeiss Microimaging Inc.). Six 10-μL drops of the suspension were added to a microscope slide to create a hanging bolus. Boli were analyzed as a mosaic 2 × 3 grid. The mosaic capture program compiled equally sized square composite images that represented the entire center of the circular bolus. Following capture, all individual images from the mosaic were analyzed using a semiautomated macro (Image) computer software. NIH, Bethesda, MD where relative fluorescent cell numbers could be determined per group/embryo.

Generation of shRNA-modified cell lines

GIPZ lentiviral shRNA particles targeting human PAPSS1 (Target Sequences: GTCTGGACATGTCTCCTAA, ACAAGTTTCA-TATCATCCT, GATCGATTCGAAATGAA) were obtained from Thermo Fisher Scientific. A549 cells were transduced using the GIPZ lentiviral shRNA starter kit (VGH5526 Thermo Fisher Scientific) as per the manufacturer’s instructions, selected with 1.5 μg/ml puromycin, and sorted into single cells using FACS at the Flow Cytometry Core Facility (Terry Fox Laboratory, British Columbia Cancer Research Centre). Please refer to Supplementary Data for detailed transduction conditions and screening procedures. The clone that was isolated, propagated, and eventually used for the murine xenograft study was derived from transduction with the PAPSS1-target sequence GATCGATTCGAAATGAA. A nonsilencing control cell line (shSCR) was generated in parallel with the PAPSS1-silenced cells. The shRNA-modified cells were used for the murine xenograft studies described below.

Murine xenograft models

All mouse xenograft studies were conducted in male SCID-RAG2 mice (6–10 weeks old, 19–25 grams) obtained from the British Columbia Cancer Agency Joint Animal Facility breeding colony. The solid tumor model was conducted by implantation of 5 × 10^6 parental, negative control shSCR, or shPAPSS1 A549 cells subcutaneously into the flank in a volume of 100 μL (n = 8). At 7 days following cell inoculation, the mice were treated intravenously with 3 mg/kg cisplatin every fourth day for a total of three doses.

Mice were monitored daily and body weight changes and signs of stress (e.g., ruffled coat and lethargy) were used as indicators of toxicities (see Supplementary Data for additional details). Tumor size was measured using an electronic caliper (541000671; Fowler High Precision) and the tumor volumes were calculated ([l × w^2]/2). Animals with ulcerated tumors or whose tumors exceeded 500 mm^3 were euthanized. Animals with multiple tumors or tumors attached to the muscles were excluded from the analysis. All tumors were collected and snap frozen at ~80°C for immunoblotting purposes.

All murine studies were completed under an animal care protocol (A14-0290) approved by the Institutional Animal Care Committee (IACC). The IACC for studies conducted at the BC Cancer Agency (Vancouver Cancer Research Centre) Animal Resource Center is operated by the University of British Columbia in accordance to CCAC.

Survival analyses

Survival comparisons for patients with low and high PAPSS1 (probe ID: 209043_at) tumor expression subsets were performed using the Kaplan–Meier Plotter platform (http://kmplot.com/; ref. 16). For NSCLC, low and high PAPSS1
expression groups were automatically determined using the best performing threshold as previously described and overall survival differences determined for each group (17). Analyses were done with all NSCLC histologic subtypes and adenocarcinoma separately, with all patients and only those undergoing adjuvant chemotherapy assessed separately in both instances. Ovarian cancer analyses were performed in a similar manner except progression-free survival was used (18). All data calculated in Kaplan–Meier Plotter was downloaded and GraphPad Prism was used to plot and compare survival curves using the log-rank test.

Statistical analyses
All data were plotted using the Prism 6.0 (GraphPad Software) as mean ± SEM unless otherwise stated. Statistical analyses were performed using Prism 6.0. Two-way ANOVA followed by Sidak adjustments for multiple test comparisons was used to analyze changes in spheroid size and A549 cell numbers in zebrafish embryos. Statistical significance of the differences tumor volumes was determined by analyzing tumor size at the final time point using one-way ANOVA followed by Tukey adjustments for multiple test comparisons. The log-rank test was used to compare the Kaplan–Meier curves between the nontargeting shRNA controls and the shPAPSS1 group. In all cases, an adjusted $P < 0.05$ was considered statistically significant.

Results
Low PAPSS1 expression is associated with higher sensitivity to cisplatin in lung adenocarcinoma cell lines
Our previous study investigated the effects of modulating PAPSS1 activity on cisplatin sensitivity in four NSCLC cell lines (6). To further validate this association, we first aimed to expand our analysis to a larger panel of lung cancer cell lines. Using data from the Sanger Institute’s Genomics of Drug Sensitivity in Cancer initiative (http://www.cancerrxgene.org/), we assessed the effects of PAPSS1 transcript levels on cisplatin IC$_{50}$ values for 52 lung adenocarcinoma cell lines. First, the average PAPSS1 expression was used to categorize the cell lines into low and high PAPSS1-expressing groups and the corresponding cisplatin IC$_{50}$ values were compared (Fig. 1A). The median cisplatin IC$_{50}$ for the low PAPSS1-expressing group was 2.4-fold lower than that of the high PAPSS1-expressing cell lines, a statistically significant difference ($P = 0.011$; Mann–Whitney $U$ test). Second, assessing PAPSS1 expression and cisplatin IC$_{50}$ across all 52 cell lines demonstrated that low PAPSS1 expression is significantly correlated with increased sensitivity to cisplatin [i.e., lower IC$_{50}$ values; Pearson correlation coefficient $= 0.244; P < 0.05$; Fig. 1B]. This association was further validated using independent data from both The Cancer Therapeutics Response Portal (Supplementary Fig. S1A) and experimental cisplatin dose–response analysis of a panel of cell lines with high and low PAPSS1 expression (Supplementary Fig. S1B and S1C). These data, combined with our previous experimental systems, strongly suggest that PAPSS1 plays an important role in regulating response to cisplatin.

PAPSS1 knockdown sensitizes NSCLC cells to cisplatin treatment under stressed conditions
It is important to address whether the potentiation effects observed with PAPSS1 knockdown are maintained when cells are deprived of oxygen and/or nutrients. A549 cells were conditioned to serum starvation, chronic hypoxia (1.0% O$_2$), or are deprived of oxygen and/or nutrients. A549 cells were conditioned to serum starvation, chronic hypoxia (1.0% O$_2$), or both, prior to and throughout drug treatment. It is evident from Fig. 2A that after 48 hours of cisplatin treatment, PAPSS1-silenced cells were more sensitive than nonsilenced cells to cisplatin treatment in all tested conditions. The potentiation effects of PAPSS1 silencing observed in hypoxia were similar to those observed under stress-free (21% oxygen, 10% serum) conditions. Under all tested stress conditions, the sensitization effects of PAPSS1 knockdown were greater compared

Figure 1.
Low PAPSS1 expression is associated with sensitivity to cisplatin in lung adenocarcinoma cell lines. Lung adenocarcinoma cell lines were separated into low and high PAPSS1-expressing groups based on gene expression microarray data (with the average PAPSS1 expression across the cell lines used to separate the groups) and cisplatin IC$_{50}$ compared using the Mann–Whitney $U$ test (box plots with whiskers indicating 10%–90% range, A). Expression of PAPSS1 was correlated with cisplatin IC$_{50}$ across all lung adenocarcinoma cell lines with the resulting Pearson correlation coefficient and $P$ value indicated (B).
with stress-free cells following 72 hours of drug exposure as demonstrated by the greater leftward shifts in the cisplatin dose–response curves (Fig. 2B). This was further highlighted when comparing the averaged fold-reduction in IC\textsubscript{50} values of PAPSS1-silenced cells relative to the scramble transfection control (Fig. 2C). The greatest potentiation effects were observed when the cells were both serum-starved and exposed to low oxygen levels for 72 hours: a 50-fold reduction in the cisplatin IC\textsubscript{50} (Fig. 2B and C) was observed when PAPSS1 was silenced (Fig. 2D). We saw no association between PAPSS1 expression and major oncogenic mutations or p53 status in lung adenocarcinoma tumors (Supplementary Fig. S2). However, the effects of hypoxia and starvation were determined in another adenocarcinoma cell line H358, which unlike A549 cells are p53 deficient (Supplementary Fig. S3). In these cells, serum starvation alone was associated with enhanced cisplatin potentiation compared with cells cultured under normal conditions.

PAPSS1-silenced cells form spheroids that are more sensitive to cisplatin treatment

We first evaluated whether PAPSS1-silenced cells (>90% reduction in mRNA expression) were capable of forming spheroids. A549 cells transfected with PAPSS1-targeting siRNAs formed spheroids over a period of three days, the same time-frame required for cells transfected with the nonsilencing control. Spheroid formation was reproducible and the spheroid sizes were comparable with the scramble control (approximately 350–400 μm in diameter; Fig. 3). On the basis of changes in fluorescence intensity during spheroid formation, PAPSS1-silenced cells appeared to be proliferating at a slower rate (Fig. 3A), with the assumption that the red fluorescence intensity was proportional to cell number. The spheroids were treated with low (1.56 μmol/L) or high (12.5 μmol/L) doses of cisplatin and were left undisturbed for 8 days. At both treatment doses, PAPSS1-depleted spheroids were more susceptible to cisplatin (Fig. 3B and C); however, the differences in spheroid size, based on fluorescence area, at the high dose were not statistically significant when scrambled controls were compared with the PAPSS1-depleted spheroids. At the low dose of cisplatin, PAPSS1-depleted spheroids were significantly more sensitive than the control (Fig. 3B, P < 0.0001). Representative images of the spheroids under each condition (displayed in Fig. 3C) show the nuclei labeled red and nonviable cells labeled with Sytox Green. The change in spheroid size was not statistically different between the two types of spheroids when left untreated. Following treatment with low-dose cisplatin, the

**Figure 2.** PAPSS1-silenced cells are more sensitive to cisplatin treatment when starved and/or exposed to low oxygen levels. Cells were reverse transfected and then acclimatized overnight to the indicated stress conditions the following day. Cisplatin was subsequently diluted in the appropriate media, and the cells were treated for 48 or 72 hours under stress. A normoxic, unstressed control was completed in parallel. The cisplatin dose–response curves for 48 hours (A) and 72 hours (B) are plotted as mean ± SEM from three independent experiments. The dose–response curves were normalized to the no-cisplatin control for each condition such that a fraction affected (Fa) of 1 represents 0% viability and Fa = 0 suggests 100% cell viability. The IC\textsubscript{50} values were interpolated and the fold reduction in the IC\textsubscript{50} of the PAPSS1-silenced cells relative to the scramble control under each test condition are plotted with error bars representing 95% confidence intervals (C). PAPSS1 knockdown was confirmed via Western blotting (D).
control spheroids grew approximately 37% in size (fluorescence area) while the PAPSS1-silenced spheroids shrank by about 21% (Fig. 3C) by day 8. These results were further supported by quantitative comparison based on viable cell counts (red fluorescence intensity) where PAPSS1-silenced spheroids treated with low-dose cisplatin had a 25% reduction in fluorescence intensity compared with the 21% increase observed with the negative control spheroids (Supplementary Fig. S4). When exposed to 12.5 μmol/L of cisplatin, the spheroid size decreased by 31% and 46% for scramble and PAPSS1-silenced spheroids, corresponding to a reduction in fluorescence intensity by 74% and 80%, respectively. To verify these findings further, we completed spheroid studies in H358 cells as well (Supplementary Fig. S5). These cells, however, were not fluorescently labeled and hence, the quantitative analysis was less precise. Nonetheless, based on spheroid diameter, we observed a 31% and 45% reduction in PAPSS1-depleted spheroids that were treated with 6.25 and 12.5 μmol/L cisplatin, respectively. Comparatively, the nonsilenced spheroids only had a 10% and 15% reduction in spheroid diameter at the same doses. These data further validate our observation that the sensitization effects observed in monolayer cultures can be observed in 3D cultures, which are more representative of the tumor structures in vivo.

PAPSS1 silencing sensitizes NSCLC cells to cisplatin treatment in zebrafish xenografts

To assess the potentiating effects of PAPSS1 knockdown on cisplatin activity against A549 cells in vivo, a zebrafish xenotransplantation model pioneered in the Berman laboratory (19) was used (results summarized in Fig. 4). Injected embryos were dissociated at 48 hpi to determine baseline A549 cell numbers as a reference for comparison with later dissociation time points. At 96 hpi, a 1.93-fold increase in cell number for both A549 scramble control and PAPSS1-silenced cells was observed in the absence of drug, demonstrating that the cells survived and proliferated in vivo. Toxicity curves for cisplatin in zebrafish were generated whereby nonxenotransplanted Casper embryos were
PAPSS1 silencing sensitizes NSCLC cells to cisplatin treatment in zebrafish xenografts. Zebrafish embryos were inoculated with scramble or PAPSS1 siRNA-transfected A549 cells and treated with different doses of cisplatin. The representative brightfield and fluorescent images at 0 and 48 hpt are shown for each condition (A). Untreated tumors showed a doubling in cell number at 48 hpt (B). The xenografts were treated with an ineffective dose of cisplatin (0.125 mmol/L), which causes no significant change in cell number in the scramble control when compared with the untreated control as well as an effective (P = 0.008) but noncurative dose (0.25 mmol/L). Cisplatin treatment at both concentrations were effective against PAPSS1-silenced cells (P < 0.005) compared with untreated controls. At both doses, PAPSS1-deficient cells were more sensitive to cisplatin treatment relative to the scramble controls (**, P < 0.01; ****, P < 0.0001). All data are plotted as mean ± SEM from three independent experiments.

PAPSS1 knockdown delays growth of tumors in mice treated with cisplatin

A549 cells expressing PAPSS1-targeting shRNAs were inoculated subcutaneously into mice. Although the parental cell line appeared to grow faster than the shRNA-modified cell lines, the differences were not statistically significant (Fig. 5A). An aliquot of the inoculated cells was probed for PAPSS1 expression via Western blotting (day 0, Fig. 5B) and the same cell population was left in culture with no selective pressure and sampled again at 7 days postinoculation for PAPSS1 protein expression (day 7, Fig. 5B). PAPSS1 expression in this modified cell line was reduced by 50% and 46% (compared with nonsilencing control cells) on day 0 and day 7, respectively. However, PAPSS1 expression in tumors (300–400 mg) arising after inoculation of the shRNA-modified cells was comparable with the control (shSCR cell line). Although this was a clonally selected cell line, these data suggest that there was a growth disadvantage for cells expressing PAPSS1-targeting shRNAs, allowing cells expressing normal levels to take over. On the basis of these results, the in vivo validation study was designed using a treatment schedule that started at a time when it was confirmed that PAPSS1 expression was low (~50%). Cisplatin treatment was initiated 7 days following cell inoculation using a Q4D × 3 dosing schedule and the dose selected (3 mg/kg) was determined to be effective at slowing the rate of tumor growth in animals inoculated with the parental cells (Fig. 5C). The results of these studies, presented in Fig. 5D and E, indicated that tumor growth following cisplatin treatment was similar between the parental and the nontargeting A549-shSCR groups. The activity of cisplatin (3 mg/kg given Q4D × 3) was greater in mice injected with the cells modified with PAPSS1-targeting shRNA (A549-shPAPSS1). The mean tumor volume of the shPAPSS1 group on day 45 was 62% (P = 0.008) and 58% (P = 0.026) lower than the parental and scramble controls, respectively, suggesting an approximately 2.5-fold reduction in tumor volume. To assess treatment efficacy, the time required for the tumors to reach 200 mm³ was determined and plotted in Fig. 5E as a Kaplan-Meier curve. This confirmed the results demonstrating enhanced efficacy of cisplatin treatment in cells with suppressed PAPSS1 expression (P = 0.0027 relative to scramble control).

Low PAPSS1 expression is associated with better survival outcomes for cancer patients treated with platins

Finally, to explore the clinical relevance of PAPSS1, we aimed to explore the impact of PAPSS1 levels on response to platinum-based
chemotherapy. We compared survival data from lung adenocarcinoma, NSCLC, and ovarian cancer patients, for which platin-based chemotherapy regimens are commonly employed, categorizing them based on PAPSS1 expression levels using Kaplan–Meier Plotter software (refs. 16, 17; Fig. 6). The top panels show the data collected from all patients, treated and untreated, while the bottom panels are data from patients who were treated with adjuvant chemotherapy. Patients with lung adenocarcinoma and NSCLC (comprises lung adenocarcinoma, large cell carcinoma, and squamous cell carcinoma) appear to have longer overall survival (OS) with higher PAPSS1 expression when treatment status was not specified [median survival 112.7 vs. 69 months and 73.3 months vs. 55.37 months for high vs. low PAPSS1, hazard ratios (HR) for low PAPSS1 = 1.47 and 1.20; P = 0.0019 and 0.0129 respectively; Fig. 6A and B, top]. However, there was a switch in that trend where lower PAPSS1 expression was associated with improved OS (median survival 6.03 months vs. 42.38 months and 29 months vs. 54.2 months for high vs. low PAPSS1, HR = 0.12 and 0.69; P <0.0001 and p = 0.0652 for lung adenocarcinoma and NSCLC, respectively; Fig. 6A and B, bottom) when only assessing the subset of patients treated with adjuvant chemotherapy (20–22). In ovarian cancer, patients with low PAPSS1-expressing tumors appear to have longer progression-free survival (PFS; median survival 22.5 months vs. 18.93 months, HR = 0.85; P = 0.012), and were almost universally receiving platinum-based treatment. However, this trend was even greater when only the subset of patients who underwent platinum-containing treatments were included in the analysis (median survival 22.57 months vs. 18 months, HR = 0.79; P = 0.0004; Fig. 6C).

Discussion

PAPSS proteins are enzymes that produce the biologically active sulfate substrate for all sulfonation reactions (23).
Although the importance of PAPSS enzymes in human physiology is becoming more apparent in recent years, the role of sulfonation in cancer and other human diseases is still poorly understood, particularly in the context of sulfonation reactions within the nucleus that rely on PAPSS1, the nuclear isoform (24–26). Bruce and colleagues have uncovered PAPSS1 as a novel target for HIV infections and have speculated that sulfonation may play a role in epigenetic modifications of DNA in the nucleus (27). In the context of cancer, we discovered PAPSS1 as a cisplatin-potentiating therapeutic target in a genome-wide siRNA screen conducted in the lung adenocarcinoma A549 cell line (6). This sensitization effect was subsequently confirmed in four different NSCLC cell lines (A549, H460, H358, H1703) of a variety of genetic backgrounds (KRAS-mutant and wild-type; P53-null, mutant, and wild-type) and histologic subtypes (adenocarcinoma, large cell carcinoma, and squamous cell carcinoma). Sensitization was not observed in PAPSS1-silenced normal bronchial epithelial cells, suggesting that PAPSS1 could be a cancer-specific cisplatin-sensitizing target. Furthermore, PAPSS1 silencing sensitized NSCLC cells to multiple DNA-damaging agents including radiation, platinum, and non-platinum–based DNA cross linkers, topoisomerase I inhibitors, and radiation. The combination of PAPSS1 inhibition with these treatment agents resulted in greater accumulation of DNA damage that ultimately lead to cell death, suggesting that sulfonation might be involved directly or indirectly with the recognition of DNA damage or DNA damage repair. It is important to note that the potentiation effects of PAPSS1 suppression on cisplatin and carboplatin activity have also been validated in several ovarian cancer cell lines using colony formation assay, suggesting that the potential for PAPSS1 to be a therapeutic target is not limited to NSCLC (25).

In the current study, we first analyzed a larger panel of cell lines (52 lung adenocarcinoma cell lines) to determine whether PAPSS1 expression levels were associated with inherent sensitivity to cisplatin. As predicted, low PAPSS1 expression was correlated with high sensitivity to cisplatin treatment. This result was consistent with our previous findings that suppressing PAPSS1 expression could enhance the cytotoxic effects of platinum-based treatments, regardless of genetic background (6). Analyzing these publicly available data also gave us stronger confidence that PAPSS1 is a relevant oncology target. It is known that tumor cells that receive suboptimal or sublethal doses of chemotherapy often reside in areas where oxygen, nutrient, and drug penetration are limited (7, 28). These are the cells that would benefit the most from potent PAPSS1 inhibition. We therefore investigated whether PAPSS1-engendered sensitization to cisplatin could be achieved in cells subjected to hypoxic and/or low nutrient conditions and in 3D spheroids in vitro as well as in vivo.

The results obtained in monolayer cultures under stress-free conditions were consistent with our previous report showing

Figure 6. Low PAPSS1 expression is associated with better survival outcomes for cancer patients treated with platin-based chemotherapy. Kaplan-Meier plots for patients with tumors with high (red line) and low (black line) expression of PAPSS1 as determined by gene expression microarrays. Plots are presented for either all patients (top row) or only the subset who underwent chemotherapy (bottom row) for each cancer type indicated: lung adenocarcinoma (A), NSCLC (B), and ovarian cancer (C). Log-rank P values and HRs with 95% confidence intervals corresponding to low PAPSS1 expression are indicated for each plot.
an approximate 5-fold reduction in the cisplatin IC₅₀ in PAPSS1-silenced A549 cells that were treated for 72 hours (6). Importantly, when PAPSS1-silenced A549 cells were serum-starved, oxygen-deprived, or stressed by both conditions, they demonstrated a log-range more sensitivity to cisplatin treatment compared with the same cells that were stress-free (normoxic, 10% serum). The potentiation effect increased from 5-fold under stress-free conditions to 50-fold in hypoxic and serum-starved cells. The trends displayed in A549 cells were similar to those in H358 cells (Supplementary Fig. S3). H358 cells are more sensitive to transfection reagents, resulting in less optimal PAPSS1 knockdown that might have contributed to the more modest sensitization effects (~2-fold reduction in IC₅₀) among other factors such as genotype and doubling time. In both cell lines, starvation was associated with enhanced cisplatin sensitization compared with PAPSS1-silenced cells cultured in normal condition. Hypoxia had no effect on nonsilenced A549 cells in terms of cisplatin sensitivity but the same conditions was associated with a 2-fold increase in IC₅₀ in nonsilenced H358 cells. Cisplatin potentiation was still observed in PAPSS1-silenced H358 cells when subjected to hypoxia, but to a lesser, nonsignificant extent compared with cells cultured in normal conditions. We have previously shown that the extent of cisplatin sensitization is strongly correlated with the level of PAPSS1 suppression at the protein level (6) and the optimal level of silencing may not be achievable using this mode of transfection for this particular cell line. In this study, we have demonstrated that the sensitization effects were similar in growth of both NSCLC cells that were subjected to oxygen and/or nutrient deprivation. It appears that the effects of oxygen and nutrient deprivation on the level of cisplatin potentiation was dependent on the level of PAPSS1 suppression as well as cell line-specific responses to various stress factors. We argue that future studies should focus on the use of potent small-molecule inhibitors of PAPSS1 to confirm these findings in a larger panel of NSCLC cell lines. This will reduce issues related to cell line-specific transfection efficiencies.

To model the tumor microenvironment in vitro, multicellular spheroids of A549 cells were grown with and without PAPSS1 depletion. Spheroids have been used to predict in vivo drug sensitivity by modeling the three-dimensional structures of tumors in vitro as a screening procedure to eliminate drug candidates that are unlikely to succeed in vivo (29–31). Spheroids consist of an outer layer of actively proliferating cells, an intermediate zone of hypoxic, viable cells, and an inner necrotic core (29, 32). Their growth characteristics and sensitivity to anticancer agents are believed to be much more representative of tumors in vivo than monolayer cell cultures (31, 33). In these spheroid studies, the A549 cells expressed a red-fluorescent protein that was used as a proliferation marker. When evaluating spheroid formation of PAPSS1-silenced cells, reduced fluorescence intensity was observed relative to the controls, suggesting decreased proliferation or increased cell death (see Fig. 3). This is consistent with our previous studies showing that PAPSS1 knockdown alone affects long-term cancer cell viability (6, 25), an effect that is not reflected in short-term cytotoxicity assays performed with monolayer cultures. Although PAPSS1-silenced cells consistently formed spheroids, it is possible that the spheroids formed were not exactly the same as those formed from the nonsilencing controls. At the start of cisplatin treatment, PAPSS1-silenced spheroids and control spheroids were comparable in size. PAPSS1 depletion sensitized cells to low-dose cisplatin in the spheroid model, while high-dose cisplatin continued to be effective against both PAPSS1-silenced and scramble spheroids (Fig. 3). Our spheroid studies were replicated in another cell line (H358), where cisplatin potentiation was apparent in PAPSS1-depleted spheroids albeit the quantitative analyses were less reliable because these cells were not fluorescently labeled (Supplementary Fig. S5). Nonetheless, data generated from both cell lines were consistent with our belief that we have uncovered a “synthetic sick” interaction where PAPSS1 inhibition impairs the fitness of the cancer cells, which in turn leads to increased sensitivity to doses of cisplatin that would not normally provide therapeutic benefits (34, 35).

To validate these results in vivo, PAPSS1 was further explored as a therapeutic target in vivo. Zebrafish models have become a powerful tool for rapid and cost-effective validation of anticancer therapies and the zebrafish xenograft model described here was previously used to graft human leukemia and sarcoma cell lines into embryos (15, 19, 36, 37). In this study, PAPSS1-silenced and nonsilenced cells proliferated at similar rates in the zebrafish embryos (see Fig. 4). The xenografts derived from PAPSS1-deficient cells were more sensitive to low doses of cisplatin (0.25 mmol/L and 0.125 mmol/L) compared with the controls, providing initial in vivo validation of in vitro findings. However, the results presented using this model are somewhat limited as growth was compared at essentially one time point, equivalent to when the controls doubled in size. Therefore, we also determined whether PAPSS-1 silencing influenced treatment responses in mouse xenografts using shRNA-modified cells. Under selection conditions, cells expressing shPAPSS1 did not regain gene expression over multiple passages. However, when established tumors were harvested from animals inoculated with these shRNA-modified cells, the expression of PAPSS1 was comparable with controls (Fig. 5). This compromised the design of the experiment and forced us to evaluate the effect of cisplatin treatment at a time when PAPSS1 suppression was confirmed (day 7) in the absence of selective pressure. The results were consistent with the preceding data and clearly indicate that the tumors derived from cells with depleted PAPSS1 are more sensitive to cisplatin treatment than the nonsilencing controls (Fig. 5), demonstrating a 2.5-fold difference in efficacy in vivo based on average tumor volumes. We believe that this level of improvement in treatment efficacy could be meaningful given that the same efficacy could be achieved at half the dose of cisplatin, which could mean a substantial reduction in the severe toxicities known to arise when using cisplatin, such as nephrotoxicity and hearing loss. Inhibition of PAPSS1 in conjunction with cisplatin treatment could have meaningful effects on the quality of life of patients receiving this drug. Given the novelty of this target and the general lack of understanding of the role of PAPSS1 and nuclear sulfonation reactions, we are the first group to establish the clinical significance of PAPSS1 in NSCLC patients. Starting with lung adenocarcinoma alone, we found that higher PAPSS1 is associated with improved OS. However, when we analyzed data from chemotherapy-treated patients only, the trend was reversed: patients with low PAPSS1 expression had a higher OS rate. Given that platinum-based chemotherapy is given in the adjuvant setting for NSCLC, it can be presumed that these patients were treated with cisplatin or carboplatin (20–22). Because of the limited number of patients in this dataset, the
findings were expanded to all NSCLC patients, where a similar trend reversal was observed. It is important to note that despite the rapid advances in cancer genomics and personalized medicine, there is still a significant lack of data on treatment response as a function of individual gene expression. Indeed, ERCC1 deficiency, which is associated with response to cisplatin-based adjuvant chemotherapy, remains one of the only such validated biomarkers in NSCLC to date (38). Our novel finding that PAPSS1 is frequently deleted at the copy number level and underexpressed in tumors with such alterations, further suggests that PAPSS1 may serve as biomarker to predict response in NSCLC, particularly in lung adenocarcinoma which demonstrates lower expression compared with squamous cell carcinoma (Supplementary Fig. S6). To further establish the potential of PAPSS1 being a clinically relevant target, we explored data collected from ovarian cancer patients as platinum-based chemotherapy is also the mainstay treatment for this patient population. Interestingly, low PAPSS1 expression was associated with longer progression-free survival regardless of their treatment status. This correlation appears to be stronger in platinum-treated patients. These results suggest that PAPSS1 expression could be a predictive marker for NSCLC and a potential prognostic and predictive marker for ovarian cancer.

In summary, we have demonstrated that PAPSS1 expression is relevant to cisplatin sensitivity in lung adenocarcinoma cells and have validated this potentiation effect in in vitro and in vivo models that resemble tumors grown in patients. We are also the first to show that low PAPSS1 expression may be associated with improved treatment outcomes in NSCLC and ovarian cancer patients. While a larger sample size would be ideal, based on the findings with existing patient gene expression and treatment response data, low expression of PAPSS1 in a tumor could be used as a predictive marker for more favorable treatment response to platinum-based chemotherapy. From a therapeutic perspective, what is not clear at this stage is the exact mechanism(s) responsible for enhanced sensitivity, but we argue that this must be associated with changes in sulfonated proteins within the nucleus and some of these proteins are important to DNA repair signaling pathways (6). Further studies have been initiated to establish whether chemosensitization is due to loss of one or both enzymatic functions of PAPSS1. A small-molecule inhibitor screen will be completed to identify small molecular weight inhibitors of the ATP sulfurylase and kinase functions of this protein. Alternatively, the effects may not be due to the loss of PAPSS1 enzymatic activity but instead result from the loss of specific protein–protein interactions (39). To address this, we hope to use tandem affinity purification assays to identify PAPSS1-binding partners (40). Such interactions, if confirmed to be causal of chemosensitization, could then be targeted with peptide therapeutics that block the protein–protein interactions (41). We believe that a small molecule or peptide targeting PAPSS1 will have broad applications in a wide range of cancers that are currently treated with cisplatin and other DNA-damaging agents as part of standard of care.

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In Vivo Validation of PAPSS1 (3′-phosphoadenosine 5′-phosphosulfate synthase 1) as a Cisplatin-sensitizing Therapeutic Target


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